Action of AlGaInP laser and high frequency generator in cutaneous wound healing. A comparative study

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ABSTRACT

PURPOSE: To evaluate in a macroscopic, histological and histomorphometric manner the healing process of cutaneous wounds in mice.

METHODS: The sample consisted of 40 male mice and was divided in four groups: 1st group (control, n=10), 2nd group (High Frequency Generator - HF, the maximum amplitude range, 120s, n=10), 3rd group (AlGaInP Laser 660 nm, 30mW power, 5 J/cm², applying scan mode, 120s, n=10) and 4th group (AlGaInP Laser 660 nm, 30 mW power, 8 J/cm², applying scan mode, n=10). The surgical incision was made with an 8 mm diameter punch perpendicularly to the back of the animal. The statistical analysis was achieved by the statistical test One Way Anova post hoc Tukey Test and significance at p<0.05 in GraphPad Prism program.

RESULTS: It was observed that in the acute phase the AlGaInP Laser at 5 J/cm² provided a greater stimulus to healing, and both lasers were effective in the remodeling phase.

CONCLUSION: The AlGaInP lasers from 5 J/cm² to 8 J/cm² showed better biomodulatory results in the acute and remodeling phases respectively, however, the HF was less effective than the laser, providing significant benefits only in the acute phase of tissue repair.

Key words: Wound Healing. Skin. Lasers, Gas. Ozone. Mice.
Introduction

The skin is an organ responsible for the interface of communication between the body and the organism and it adapts to perform the most diverse functions, like coating, thermoregulation, secretion and defense. However, some events can lead to tissue loss and cause wounds, which constitutes a break in the continuity of a corporal tissue, whose causes are: physical, chemical or mechanical traumas among others. In this context, there is stimulation of the organism’s defense and the beginning of the cicatrization, which consists in reconstructing the injured site, in order to restore the integrity and normal function, besides being complex and dynamic, and involving biochemical and physiological events, which must act in harmony for the benefit of tissue restoration.

However, the complex process of tissue cicatrization can be influenced, it may not occur in a successful manner and it has long been an object of study concerning the factors that affect it. In recent years, researchers have used different equipments to facilitate the healing process of skin wounds with demonstrably scientific results. In this context, it refers to the low power laser (LPL), as AlGaInP, the AsGa and HeNe, therapeutic ultrasound, LED (light emitting diode) and the High Frequency Generator (HF) as current resources that, according to the literature, show beneficial and effective action.

In the last years, several studies have shown the benefits of the extensive use of the LPL in situations such as the treatment of soft tissue injuries, joint diseases, open wounds, pressure ulcers, among others. The AlGaInP laser (660 nm) is a therapeutic light of the electromagnetic spectrum in the red line, whose irradiation in injured tissues triggers a series of physiological effects necessary for the process of wound cicatrization and repair at tissue and organic levels by means of the photobiomodulation process, in addition to causing therapeutic effects, such as: local analgesia, edema reduction and anti-inflammatory action.

Another relevant resource for the tissue repair process is the HF - product of an alternating current of high frequency and low intensity, widely used in aesthetics. This resource uses ozone as a therapeutic agent, whose potent oxidizing effect stimulates biochemical events during cellular metabolism, besides having antimicrobial, bactericidal and fungicidal effects.

Studies show that the LPL, as well as HF, accelerate the tissue repair process for wounds, after incisional lesion, being relevant in the treatment of skin disorders, once the AlGaInP laser reduces the inflammatory reaction and provides better quality and greater speed in the repair process of soft tissue, while the HF can intervene in the oxidoreduction balance with germicide, bactericide and antiseptic properties in the cicatrization process. Given this, the goal was to have a macroscopic, histological and histomorphometric assessment of the process for cutaneous wound cicatrization in mice, by using AlGaInP Laser therapy compared to treatment with HF.

Methods

The study was approved by the Animal Ethics Committee - AEC of FACID/DeVry with the protocol No. 029/13, on 12.19.2013, according to the Arouca Federal Law No. 11.974/2008 - Animal Experimentation.

Experimental groups

The sample was composed by forty male mice (Mus musculus, Muridae Family) with a mean weight between 20 and 30 grams, randomly divided into four groups according to the treatment and subdivided into two groups (A and B), according to the period of observation after the treatment, of seven and 14 days. The experimental groups were:

- Group 1: control (n= 10).
- Group 2: High Frequency Generator - HF (maximum amplitude band, cautery electrode, 120 s) (n= 10).
- Group 3: laser AlGaInP (660nm), applying scan mode, 30 mW power, 5 J/cm², 120 s (n= 10).
- Group 4: laser AlGaInP (660nm), applying scan mode, 30 mW power, 8 J/cm², 120 s (n= 10).

Induction of experimental injury

The animals were weighed and then anesthetized with the administration, by intramuscular injection, of ketamine hydrochloride 10% in dose of 0.1 ml per 100g of body weight associated to the same dose of xylazine hydrochloride 2%. The anesthetic drugs were applied independently. The skin wound was induced in the back of the animals, in a dorsal decubitus position, initiated with the trichotomy of the region. To perform the experimental injury a surgical instrument (punch) of 8 mm in diameter was utilized, perpendicularly positioned to the back, of which the wound depth was corresponding to the epidermis and dermis layers of the skin.
Treatment of cutaneous wound

The first AlGaInP laser irradiation at 5 J/cm²; AlGaInP at 8 J/cm² and the application of HF occurred 24 hours after the injury for groups 2, 3 and 4, respectively. The animals of subgroup A (euthanized on day 7) received their treatment corresponding to five times, while the animals of Subgroup B (euthanized on day 14) received 11 times, both applied daily, once a day in consecutive days. The animals were accommodated in polypropylene cages and maintained during the whole experiment in good conditions of hygiene, temperature and illumination, and fed on standard diet of the vivarium, food (Labina™) and water “ad libitum”.

The study was carried out using a low power laser AlGaInP (Laserpulse Ibramed) with a continuous wavelength of 660 nm, 30 mW power, scan mode within the area of the cutaneous wound and with a contact area of 0.06310 cm². The High Frequency generator used for ozone generation was Ibramed HF - High Frequency Machine Facial Treatments, technique of sparking with cautery electrode (monopolar) inside the cutaneous wound area, maximum amplitude range, 50/60 Hz, 15 VA. The calibrated equipment was acquired by the researcher and used for the first time in this experiment.

The euthanasia of the animals happened on the 7th and 14th days, when there was the removal of the tissue for histological, histomorphometric and macroscopic analyzes. The mice were submitted to euthanasia by an overdose of sodium thiopental in the concentration of 50 mg/ml.

Macroscopic analysis

The areas of the injuries were registered immediately after its production, seven and 14 days of treatment through a Nikon digital camera COOLPIX L820, resolution of 16 Megapixels, fixed on a tripod, kept at a constant distance of 30 cm from the surgical area. For the determination of the wound area and image analysis, the ImageJ software was used to calculate the area of the same.

Histological and histomorphometric analysis

The surgical specimen was removed immediately after the animal euthanasia, with a margin of 1 cm of skin around the lesion. Starting with the material fixed in 10% formalin, the histological technique of routine was processed, including the steps of gradual dehydration, diaphanization, infiltration and embedment in paraffin.

The samples were submitted to longitudinal histological cuts, stained with hematoxylin eosin (HE) and Masson trichromic and analyzed by microscope Olympus CX31, with x400 magnification. A qualitative histological analysis of inflammatory reaction was made, defined by: reepithelization, granulation tissue, presence of inflammatory cells, fibroblasts, collagen deposition and neovascularization, according to criteria described in the literature. The histomorphometric quantitative analysis included the differential count of the presence of inflammatory cells, fibroblasts and blood vessels through the ImageJ software.

Statistical analysis

The D’Agostino & Pearson normality test was applied and the statistic treatment was obtained by paired comparison with the help of the parametric test One-Way Anova test post hoc Tukey. The data were normal and considered significant when p<0.05, through the GraphPad Prism 5.0 program.

Results

As shown in Figure 1, the macroscopic analysis of the percentage regression for cutaneous wound in mice at seven and 14 days, Group 3 (Laser 5 J/cm²) was the only statistically significant group when compared to the other groups on day 7; however, in Table 1, which shows a qualitative distribution of the results found in the histological analysis, all groups showed a slight reepithelialization and, as to the granulation tissue, groups 2 (HF) and 4 (laser 8 J/cm²) were found moderate and groups 1 (control) and 3, intense.

At 14 days, the laser groups of 5J/cm² and 8 J/cm² showed to be statistically higher for wound regression (Figure 1) compared to groups 1 and 2 and, considering Table 1, the reepithelialization of most part of the analyzed samples was moderate (except the group 3, which was discreet) and the granulation tissue remained predominantly moderate in the groups. That way, the results suggest that lower doses of LPL (5 J/cm²) are more effective in the acute phase of the inflammatory process, while in the tissue repair remodeling phase, the laser was effective for both doses tested.
FIGURE 1 - Analysis of the percentage of the wound regression of different groups at 7 and 14 days. Data groups were compared with an analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests.

**: significance in relation to the control group; a: statistical significance in comparison to HF group and b: statistical significance in comparison to the Laser group at 8 J/cm².

In Figures 2, 3 and 4, we obtained the histomorphometric analysis related to, respectively, the number of vessels, the presence of fibroblasts and inflammatory cells present in the different groups at 7 and 14 days.

Figure 2 shows that at seven days, Group 2 received a significantly higher amount of blood vessels than Group 3, though for the qualitative distribution of histological analysis (Table 1) the HF group (group 2) has had a moderate formation of neovascularization and the laser at 5 J/cm² (group 3) has been intense, which may be related to the fact that literature¹⁶ suggests very large intervals for histological analysis criteria. At 14 days the statistically significant results were shown in the comparison of laser groups, where the one which had employed higher doses, in this case, 8 J/cm² (group 4), proved to be more relevant histomorphometrically, yet, in the qualitative distribution of histological analysis (Table 1) all groups showed an intense neovascularization.

FIGURE 2 - Histomorphometric analysis of the number of vessels present in the different groups at 7 and 14 days. Data Groups were compared with an analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests.

a: significance of the HF in relation to laser 5J/cm²; b: significance of the laser 8 J/cm² in relation to laser 5 J/cm².
Concerning the histomorphometric analysis of the proliferation of fibroblasts (Figure 3), at 7 days all groups were effective compared to control group, especially group 4, whose results were very significant. Similar results were found in the histological analysis, where all groups found moderate presence of fibroblasts, including the control group, with a discreet presence of collagen fibers.

At 14 days only Group 3 showed statistically higher values than Group 1. Nonetheless, it should be observed that in group 4, there was a higher visible reduction in the average of fibroblasts / field between seven and 14 days because it fell from ±185.80 to ±147.60, which suggests that there was a bigger stimulus to maturation of fibroblasts proliferated in collagen for this group with higher doses of LPL irradiation (8 J/cm²) compared to others. Table 1 also showed this tendency to maturation of collagen fibers at 14 days, which went from discreet to moderate in most of the samples analyzed in almost all groups.

**TABLE 1 - Summary of results for the histological analysis (%) at 7 and 14 days.**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Control</th>
<th>HF</th>
<th>Laser 5J</th>
<th>Laser 8J</th>
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<td>7 days</td>
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<tr>
<td>Reepithelization</td>
<td>100 - -</td>
<td>100 - -</td>
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<td>Acute inflammation</td>
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<tr>
<td>Chronic inflammation</td>
<td>- 100 -</td>
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<td>100 - -</td>
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<tr>
<td>Mixed inflammation</td>
<td>- 100 -</td>
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<td>Granulation tissue</td>
<td>- 100 -</td>
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<td>- 100 -</td>
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<tr>
<td>Neovascularization</td>
<td>- 100 -</td>
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<tr>
<td>Fibroblasts</td>
<td>- 100 -</td>
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<td>- 100 -</td>
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<tr>
<td>Collagen fibers</td>
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<td>14 days</td>
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<td>Reepithelization</td>
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<tr>
<td>Collagen fibers</td>
<td>- 80 20</td>
<td>60 40 -</td>
<td>20 80 -</td>
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</tbody>
</table>

Legend: D= Discrete, M= Moderate e I= Intense.

**FIGURE 3 - Histomorphometric analysis of the fibroblasts quantity present in the different groups at 7 and 14 days. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests.

*: significant results in relation to control group; ** very significant results in relation to control group.
Regarding the presence of inflammatory cells (Figure 4), the histomorphometric analysis of the 7 days revealed that the HF groups and Laser groups at 5 J/cm² were more effective in reducing inflammation in acute phase of cicatrization than the LPL at higher doses. Although it did not obtain a significant result compared to control, on average, group 2 was the one that most shortened the inflammatory process in the early phase of the tissue cicatrization.

Similarly, in the histological analysis, at seven days, only the group that received treatment with HF showed acute and moderate mixed inflammation, while for all other groups it showed to be intense. Still considering this analysis, the laser group of J/cm² was the one which had discreet chronic inflammation and the others, moderate.

At 14 days there were no significant results, but the lowest average for inflammatory cells was found in the Laser group at 5 J/cm², as well as the greater reduction of the amount of inflammatory cells between seven and 14 days found in the Laser Group at 8 J/cm², which changed from 48.80 ± 4.89 to 30.80 ± 5.23 inflammatory / field cells. However, unlike the tendency of other groups, that on average showed a reduction in this parameter between 7 and 14 days, there was an increase in the HF group because the quantity of inflammatory cells / field went from 20.00 ± 4.27 to 35.20 ± 6.91.

**Discussion**

Studies suggest that LPL, as well as HF, facilitate tissue repair of cutaneous wounds. Similar results, especially in relation to the laser, were found in this study. Although the HF did not display a good efficacy in the comparative analysis of this study, it is known to play a beneficial role in the dehiscence of surgical wounds and pressure ulcers, since the aid in the treatment of the cutaneous lesions and infected dermatological lesions is associated with its confirmed antiseptic and bactericidal action.

In another research, which compared the treatment of HF (amplitude range of 80%, small Standard electrode 120s) with the LPL (AlGaInP, 670 nm, 30 mW, 6 J/cm², 1 point, 120s) and the combined therapy of both, the HF, as well as the LPL, showed significant results in the regression of the wound (p<0.05); the combination therapy was even more significant (p<0.01). In comparison to this study (Figure 1), in the same range of days evaluated, only the Laser group at 5 J/cm² was effective, after seven days of incisional injury, although with different doses and application mode.

Positive effects of the LPL (630 nm) also have been found in the initial and middle phases of the healing process of cutaneous wounds in diabetic mice by a dose of 3.6 J/cm² compared in different powers: 5 mW/cm² (12 min), 10 mW/cm² (6 min) and 20 mW/cm² (3 min), applied five times / week for two weeks. It was observed that all laser groups promoted significant results in wound contraction in relation to the control group on the 3rd, 6th and 9th day of treatment, but none on the 12th day.

It is observed that the low dosage used in the aforementioned study was effective only in the initial phase of wound healing when compared to control group. The present study (Figure 1), which used higher doses and power of LPL, also presented significant laser results in lower dose in the acute phase of wound cicatrization and, in both doses, the lowest and the highest, in the remodeling phase, probably because the higher doses of LPL are more efficient in the remodeling phase than in the acute phase.

Regarding the microscopic findings, a study with similar variables also proved satisfactory performance of AlGaInP laser for the burns healing treatment, because the semiquantitative histological analysis, after 10 days, showed that LPL (660 nm,
precise, continuous mode, 30 mW, 10 J/cm² for 9 seconds/point inside the burning in six points and 12 J/cm² for 11 seconds/point in the wound edge in 14 points) promoted a significant proliferation of fibroblasts, collagen, new vessels and cutaneous annexes.

Similar to such effect of LPL in burns26, in this study there was also a significant presence of histomorphometric fibroblasts for laser groups in relation to control group (Figure 3), as well as a strong presence of new vessels (at seven and 14 days) and moderate presence of collagen (at 14 days), as seen in the histological analysis of Table 1.

Another study7 showed that the LPL (InGaAlP, 660 nm, 4 J/cm², 660 nm for 24 seconds) has a positive influence on the percentage of collagen and macrophages in cutaneous wounds, increasing the average amount of collagen fibers and reducing the macrophages fibers. However, in this study none of laser groups showed lower quantities of inflammatory cells when compared to the control group in a statistically significant form. The group in which higher doses of laser were applied, achieved a significant higher amount of inflammatory cells compared to other treatment groups analyzed, probably because smaller LPL doses are more effective to reduce inflammation in the acute phase of cicatrization.

In a research using the LPL AsGa (904 nm with peak power of 15 mW) in oxidative parameters (the respiratory chain enzymes) in wound cicatrization with 18 rats, randomly divided into three groups (control 5 days, 5 days/2 J/cm², 5 days/4 J/cm²), submitted to one single circular wound of 8 x 8 mm in the mouse’s dorsum, it was concluded that the laser stimulates antioxidant activity thus protecting cells against oxidative damage during the process of cicatrization for cutaneous wounds in rats12.

According to literature, doses ranging from 3-6 J/cm² appear to be more effective, while doses higher than 10 J/cm² are associated with harmful effects11, nevertheless, it was proved that a daily dose of 30 J/cm² and power density at 25 mW/cm² of 670 nm laser, continuous mode, was able to shorten the inflammation without compromising the metabolism of fibroblasts, positively influencing all the phases of wound cicatrization in the skin of 49 rats, which produced two parallel wounds on the back, where one served as a control and the other was exposed to laser irradiation21.

In a study25 that compared the LPL irradiation at a dose of 3.6 J/cm² in different powers: 5 mW/cm² (12 min), 10 mW/cm² (6 min) and 20 mW/cm² (3 min), applied 5 times/week for two weeks, showed that, generally the larger laser irradiation powers were more effective and significant for the quality of the cicatrization process, according to some histological criteria, such as collagen synthesis, proliferation of fibroblasts, epithelialization, quantity of polymorphonuclear and neovascularization.

In general, it is observed that in the literature there is no consensus about the ideal parameters for the application of LPL on cutaneous wounds, however, it is known that its biological effect depends on three ideal parameters: wavelength, dosing, and power density21.

Conclusions

Low power laser was more effective at 5 J/cm² than at 8 J/cm² in the acute phase of the cicatrization process. For the remodeling phase, both doses presented relevant results and laser at 8 J/cm² presented more significant results at 14 days than at seven days, suggesting that in the initial phase of the cicatrization process, lower doses are more effective, whereas in the more advanced phase, higher doses will bring more stimuli to tissue regeneration.

As to the HF, although it did not present the same efficacy as the LPL, its performance was also more effective in the initial phase of cicatrization. However, for further conclusions about the effectiveness of this resource in the cutaneous wound cicatrization process, more studies are recommended to know about the factors that affect the process of repair for these tissues.

References

8. Neves SMV, Nicolau RA, Maia Filho ALM, Mendes LMS, Veloso


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